ASSAY FOR IMIDAZOLINONE RESISTANCE MUTATIONS IN *BRASSICA* SPECIES

[001] This application claims priority under 35 U.S.C. § 119(e) to U.S. provisional application serial number 60/421,994, filed October 29, 2002.

[002] This invention relates generally to compositions and methods for identifying *Brassica* plants having increased tolerance to an imidazolinone herbicide.

BACKGROUND OF THE INVENTION

[003] Canola is the seed derived from any of the *Brassica* species *B. napus*, *B. campestris/rapa*, and certain varieties of *B. juncea*. Canola oil is high in monounsaturated fats, moderate in polyunsaturated fats, and low in saturated fats, having the lowest level of saturated fat of any vegetable oil. Thus canola oil is an important dietary option for lowering serum cholesterol in humans. In addition, the protein meal which is the byproduct of canola oil production has a high nutritional content and is used in animal feeds.

[004] Imidazolinone and sulfonylurea herbicides are widely used in modern agriculture due to their effectiveness at very low application rates and relative non-toxicity in animals. Both of these herbicides act by inhibiting acetohydroxyacid synthase (AHAS; EC 4.1.3.18, also known as acetolactate synthase or ALS), the first enzyme in the synthetic pathway of the branched chain amino acids valine, leucine and isoleucine. Several examples of commercially available imidazolinone herbicides are PURSUIT® (imazethapyr), SCEPTER® (imazaquin) and ARSENAL® (imazapyr). Examples of sulfonylurea herbicides are chlorsulfuron, metsulfuron methyl, sulfometuron methyl, chlorimuron ethyl, thifensulfuron methyl, tribenuron methyl, bensulfuron methyl, nicosulfuron, ethametsulfuron methyl, rimsulfuron, triflusulfuron methyl, triasulfuron, primisulfuron methyl, cinosulfuron, amidosulfuron, fluzasulfuron, imazosulfuron, pyrazosulfuron ethyl and halosulfuron.

[005] Due to their high effectiveness and low toxicity, imidazolinone herbicides are favored for application to many crops, including canola, by spraying over the top of a wide area of vegetation. The ability to spray an herbicide over the top of a wide range

of vegetation decreases the costs associated with plantation establishment and maintenance and decreases the need for site preparation prior to use of such chemicals. Spraying over the top of a desired tolerant species also results in the ability to achieve maximum yield potential of the desired species due to the absence of competitive species. However, the ability to use such spray-over techniques is dependent upon the presence of imidazolinone resistant species of the desired vegetation in the spray over area. In addition, because residual imidazolinones persist in a sprayed field, a variety of resistant species is advantageous for crop rotation purposes.

[006] Unfortunately, the *Brassica* species which are the source of canola are closely related to a number of broad leaf cruciferous weeds, for example, stinkweed, ball mustard, wormseed mustard, hare's ear mustard, shepherd's purse, common peppergrass, flixweed, and the like. Thus it was necessary to develop *Brassica* cultivars which are tolerant or resistant to the imidazolinone herbicides. Swanson, *et al.* (1989) *Theor. Appl. Genet.* **78**, 525-530 discloses *B. napus* mutants P_1 and P_2, developed by mutagenesis of microspores of *B. napus* (cv 'Topas'), which demonstrated tolerance to the imidazolinone herbicides PURSUIT® and ASSERT® at levels approaching ten times the field-recommended rates. The homozygous P_2 mutant produced an AHAS enzyme which was 500 times more tolerant to PURSUIT® than wild type enzyme, while the AHAS enzyme from the homozygous P_1 mutant was only slightly more tolerant than the wild type enzyme. In field trials, the P_1, P_2, and P_1 x P_2 hybrid withstood ASSERT® applications up to 800 g/ha with no loss of yield. The P_1 and P_2 mutations were unlinked and semidominant, and P_1 x P_2 crosses tolerated levels of PURSUIT® higher than those tolerated by either homozygous mutant. Imidazolinone-tolerant cultivars of *B. napus* were developed from the P_1 x P_2 mutants and have been sold as CLEARFIELD® canola. *See also*, Canadian patent application number 2,340,282; Canadian patent number 1,335,412, and European patent number 284419.

[007] Rutledge, *et al.* (1991) *Mol. Gen. Genet.* **229**, 31-40) discloses the nucleic acid sequence of three of the five genes encoding AHAS isoenzymes in *B. napus*, *AHAS1*, *AHAS2*, and *AHAS3*. Rutledge, *et al.* discusses the mutants of Swanson, *et al.* and predicts that the two alleles that conferred resistance to imidazolinone herbicides

correspond to *AHAS1* and *AHAS3*. Hattori *et al.* (1995) *Mol. Gen. Genet.* **246**, 419-425 disclose a mutant allele of *AHAS3* from a mutant *B. napus* cv Topas cell suspension culture line in which a single nucleotide change at codon 557 leading to an amino acid change from tryptophan to leucine confers resistance to sulfonylurea, imidazolinone, and triazolopyrimidine herbicides. Codon 557 of Hattori, *et al.* corresponds to codon 556 of the *AHAS3* sequence disclosed in Rutledge, *et al.*, *supra*, and to codon 556 of the *AHAS3* sequence set forth as GENBANK accession number gi/17775/emb/Z11526/.

[008] A single nucleotide mutation at codon 173 in a *B. napus* ALS gene, corresponding to *AHAS2* of Rutledge *et al.*, *supra*, leads to a change from Pro to Ser (Wiersma *et al.* (1989) *Mol. Gen. Genet.* **219**, 413-420). The mutant *B. napus AHAS2* gene was transformed into tobacco to produce a chlorsulfuron tolerant phenotype.

[009] U.S.Pat.Nos. 6,114,116 and 6,358,686 disclose nucleic acid sequences from *B. napus* and *B. oleracea* containing polymorphisms, none of which appears to correspond to the polymorphism disclosed in Hattori, *et al., supra.*

[010] For commercially relevant *Brassica* cultivars, it is necessary to ensure that each lot of herbicide-resistant seed contains all mutations necessary to confer herbicide tolerance. A method is needed to detect mutations in *Brassica AHAS1* and *AHAS3* genes that confer increased imidazolinone tolerance to commercial cultivars.

SUMMARY OF THE INVENTION

[011] The present invention describes the location and identity of a single nucleotide polymorphism at position 1874 of the *AHAS1* gene of *B. napus* as set forth in Figure 1, the polymorphism being designated as the PM1 mutation. The PM1 mutation confers about 15% of the tolerance to imidazolinone herbicides that is present in CLEARFIELD® canola. CLEARFIELD® canola also contains a second single nucleotide polymorphism at position 1712 of the *AHAS3* gene of *B. napus* as set forth in Figure 2, which corresponds to the tryptophan to leucine substitution described in Hattori *et al., supra.* For the purpose of the present invention, this polymorphism is designated as the PM2 mutation. The PM2 mutation confers about 85% of the tolerance to imidazolinone herbicides exhibited by CLEARFIELD® canola. Both the

PM1 and PM2 mutations are required to produce a *Brassica* plant with sufficient herbicide tolerance to be commercially relevant, as in CLEARFIELD® canola.

[012] Accordingly, the present invention provides methods of identifying a plant having increased tolerance to an imidazolinone herbicide by detecting the presence or absence of the *B. napus* PM1 and PM2 mutations in the plant. One of the advantages of the present invention is that it provides a reliable and quick means to detect plants with commercially relevant imidazolinone tolerance.

[013] In one embodiment, the invention provides a method of assaying a *Brassica* plant for imidazolinone herbicide tolerance conferred by the PM1 mutation of the *B. napus AHAS1* gene. In this method, genomic DNA is isolated from the plant, and the *AHAS1* gene is selectively amplified from the genomic DNA using an *AHAS1* forward primer and an *AHAS1* reverse primer in a first amplification step, thereby producing an *AHAS1* reaction mixture. The *AHAS1*-specific primers are removed from the *AHAS1* reaction mixture to produce a purified *AHAS1* reaction mixture. The amplified *AHAS1* gene is then further amplified in a second amplification step to produce a portion of the *AHAS1* gene containing the site of the PM1 mutation, by combining the purified *AHAS1* reaction mixture with a PM1 forward primer and a PM1 reverse primer, wherein the PM1 forward primer and the PM1 reverse primer are nested within the *AHAS1* forward and reverse primers. The product of the second amplification step is then denatured and allowed to adopt a conformation determined by intramolecular interactions and base stacking, to produce unique single-stranded structures dependent on sequence composition, also referred to as conformers, and the presence or absence of the PM1 mutation is detected on the basis of the mobility of said single stranded structural conformers in a substrate. The detection step of this embodiment is generally known as single strand conformational polymorphism detection.

[014] In another embodiment, the invention provides a method for assaying a *Brassica* plant for imidazolinone herbicide tolerance conferred by the PM2 mutation of the *B. napus AHAS3* gene. In this method, genomic DNA is isolated from the plant, and the *AHAS3* gene is selectively amplified from the genomic DNA using an *AHAS3* forward primer and an *AHAS3* reverse primer in a first amplification step to produce an *AHAS3* reaction mixture. The *AHAS3* primers are removed from the

AHAS3 reaction mixture to produce a purified *AHAS3* reaction mixture. The amplified *AHAS3* gene is further amplified in a second amplification step by combining a first aliquot of the purified *AHAS3* reaction mixture with at least one primer selective for a portion of said *AHAS3* gene which comprises a "G" residue at position 1712 of the *AHAS3* gene as depicted in SEQ ID NOs:5 and 8, that is, the "wild type" primer is selective for an *AHAS3* gene which is inhibited by imidazolinone herbicides. The amplified *AHAS3* gene is further amplified in a third amplification step by combining a second aliquot of the purified *AHAS3* reaction mixture with a PM2 primer selective for a portion of said *AHAS3* gene containing the PM2 mutation. The amplified first and second aliquots are then analyzed for the presence or absence of the PM2 mutation.

[015] In another embodiment of the invention, presence or absence of both the PM1 mutation and the PM2 mutation in a *Brassica* plant is determined using the above-described methods.

[016] In yet another embodiment, the invention provides oligonucleotide primers for specific amplification of the *B. napus AHAS1* gene and the region of the *AHAS1* gene corresponding to the PM1 mutation, and for specific amplification of the *B. napus AHAS3* gene and the region of the *AHAS3* gene corresponding to the PM3 mutation.

[017] In another embodiment, the invention provides isolated nucleic acids produced as reaction products of the specific amplification of the *B. napus AHAS1* gene and the region of the *AHAS1* gene corresponding to the PM1 mutation, and isolated nucleic acids produced as reaction products of specific amplification of the *B. napus AHAS3* gene and the region of the *AHAS3* gene corresponding to the PM3 mutation.

[018] In another embodiment, the invention provides a method of marker-assisted breeding of canola plants using the PM1 and PM2 assays, oligonucleotide primers, and amplification reaction products disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[019] Figure 1 shows the aligned nucleic acid sequences of *AHAS1* isolated from several varieties of *B. napus* (SEQ ID NOs: 1-4). The position of the PM1 mutation is indicated (position 1874), as are the positions of preferred PCR amplification primers.

[020] Figure 2 shows the aligned nucleic acid sequences of *AHAS3* from several varieties of *B. napus* (SEQ ID NOs: 5-8). The position of the PM2 mutation is indicated (position 1712), as are the positions of preferred PCR amplification primers.

[021] Figure 3 is a diagram of one embodiment of the present invention's method for detection of the PM1 mutation.

[022] Figure 4 is a diagram of one embodiment of the present invention's method for detection of the PM2 mutation.

[023] Figure 5 shows the aligned *AHAS1* (SEQ ID NO:19) and *AHAS3* (SEQ ID NO:20) genes and the positions of the *AHAS1* forward amplification primer (SEQ ID NO:9); the *AHAS1* reverse amplification primer (SEQ ID NO:10); the *AHAS3* forward amplification primer (SEQ ID NO:13); and the *AHAS3* reverse amplification primer (SEQ ID NO:14).

DETAILED DESCRIPTION OF THE INVENTION

[024] The present invention provides methods and compositions for identifying plants having increased tolerance to an imidazolinone herbicide by virtue of the presence of the *B. napus* PM1 and PM2 mutations. More particularly, the methods and compositions of the present invention allow identification of *Brassica* seeds and plants having commercially relevant imidazolinone tolerance, such as CLEARFIELD® canola. In some embodiments, the methods of the invention employ novel polynucleotide primers including PM1 extension primers and PM2 extension primers.

[025] It is to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for example, reference to "a cell" can mean that at least one cell can be utilized.

[026] For the purposes of the present invention, the level of tolerance to imidazolinone herbicides exhibited by CLEARFIELD® canola which contains both the PM1 and PM2 mutations is defined as "100% tolerance", or "commercially relevant imidazolinone tolerance" or "commercial field tolerance". The terms "tolerance" and "resistance" are used interchangeably herein.

[027] An oligonucleotide as defined herein is a nucleic acid comprising from about 8 to about 25 covalently linked nucleotides. A polynucleotide as defined herein is a

nucleic acid comprising more than 25 covalently linked nucleotides. In accordance with the invention, oligonucleotides and ploynucleotides may comprise nucleic acid analogs, including, without limitation, phosphorothioates, phosphoramidates, peptide nucleic acids, and the like. An "isolated" nucleic acid is substantially of essentially free from components which normally accompany it as found in its native state.

[028] As defined herein, a "PM1 mutation" refers to a single nucleotide polymorphism in a *B. napus AHAS1* gene in which there is a "G" to "A" nucleotide substitution at position 1874 of the *AHAS1* wild type polynucleotide sequence shown in Figure 1, the mutation being represented in SEQ ID NOs:1 and 3, which substitution leads to a serine to asparagine amino acid substitution at position 638 in the *B. napus* AHAS1 enzyme.

[029] As defined herein, a "PM2 mutation" refers to a single nucleotide polymorphism in a *B. napus AHAS3* gene in which there is a "G" to "T" nucleotide substitution at position 1712 of the *AHAS3* wild type polynucleotide sequence shown in Figure 2, the mutation being represented in SEQ ID NOs:6 and 7, which substitution leads to a tryptophan to leucine amino acid substitution at position 556 in the *B. napus* AHAS3 enzyme.

[030] The presence of the PM1 and PM2 mutations in a plant confers tolerance to such imidazolinone herbicides as PURSUIT® (imazethapyr, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid), CADRE® (imazapic, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-methyl-3-pyridinecarboxylic acid), RAPTOR® (imazamox, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(methoxymethyl)-3-pyridinecarboxylic acid), SCEPTER® (imazaquin, 2-(4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl)-3-quinolinecarboxylic acid), ASSERT® (imazethabenz, methyl esters of 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-4-methylbenzoic acid and 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-methylbenzoic acid), ARSENAL® (imazapyr, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-pyridinecarboxylic acid), and the like. In addition, the PM1 and PM2 mutations may confer resistance to sulfonylurea, triazolopyrimidine, pyrimidinyl(thio)benzoate, and sulfonylamino-carbonyl-triazolinone herbicides.

[031] The PM1 and PM2 mutations may be present in a plant by virtue of mutagenesis of any species of plant containing the *B. napus AHAS1* and *AHAS3* genes, respectively. Alternatively, the PM1 and PM2 mutations may be present in a plant by virtue of transformation of the *B. napus AHAS1* PM1 gene and the *B. napus AHAS3* PM2 genes into the plant, using known methods such as those set forth in U.S.Pat.Nos. 5,591,616; 5,767,368; 5,736,369; 6,020,539; 6,153,813; 5,036,006; 5,120,657; 5,969,213; 6,288,312; 6,258,999, and the like. Preferably, the plant is a *Brassica* oilseed. More preferably, the plant species is selected from the group consisting of *B. napus*, *B. campestris/rapa*, and *B. juncea*. Most preferably, the plant species is *B. napus*. In accordance with the present invention, the term "plant" includes seeds, leaves, stems, whole plants, organelles, cells, and tissues.

[032] In the first step of the methods of the invention, genomic DNA is isolated from the plant. It is to be understood that when practicing the methods of the present invention, genomic DNA can be extracted from the plant by any method known to those of skill in the art. Genomic DNA can be extracted from a whole plant, a plant leaf, a plant stem, a plant seed, or any plant organelle, cell or tissue. One non-limiting method for extracting the DNA from a plant leaf is described in Example 1 below.

[033] When assaying for the presence or absence of the PM1 mutation, in the second step the *AHAS1* gene is selectively amplified from the isolated genomic DNA. Amplification can be achieved using any method known to those of skill in the art including PCR. The term "PCR" as used herein refers to the polymerase chain reaction method of DNA amplification. As will be understood by one of ordinary skill in the art, this term also includes any and all other methods known in the art for nucleic acid amplification requiring an amplification target, at least one primer and a polymerase. For example, the *AHAS1* gene, or a portion thereof which contains the site of the PM1 mutation, may be amplified by combining the isolated genomic DNA with an appropriate primer set for the amplification of a polynucleotide sequence containing a PM1 mutation. Each primer set consists of a forward primer and a reverse primer, each of which can be referred to as an "amplification primer." In one embodiment of the present invention, the *AHAS1* gene may be amplified using a primer set wherein the *AHAS1* forward amplification primer comprises the sequence 5' CACAAGTCTCGTGTTATAAAAC 3' (SEQ ID NO:9) and the *AHAS1* reverse

amplification primer comprises the sequence 5' CATTGAGTGCCAAACATATGAA 3' (SEQ ID NO:10). Those of skill in the art will recognize that other primers may be used to selectively amplify the *B. napus AHAS1* gene. As is well known, amplification occurs through cycles of incubation of the genomic DNA, the primers, a thermostable DNA polymerase, and nucleotides under conditions suitable for DNA synthesis, as described in U.S.Pat.Nos. 4,683,195; 4,683,202; 4,965,188; 5,998,143, and the like. Apparatus and reagents suitable for PCR amplification are commercially available, for example, from Applied Biosystems, Inc. Foster City, CA.

[034] After the first amplification step, the *AHAS1* amplification reaction product or mixture is purified to remove the *AHAS1*-specific amplification primers. Any method may be used for this purification step. Preferably, commercially available PCR purification methods such as the Wizard MagneSil PCR Cleanup System (ProMega, Madison, WI, USA) is used to remove the *AHAS1* amplification primers from the *AHAS1* amplification mixture. More preferably, the *AHAS1* amplification primers are removed by exonuclease digestion. Any exonuclease capable of specifically digesting single stranded DNA may be used for the digestion. For example, Exonuclease T (RNAase T), S1 nuclease from *Aspergillus oryzae*, Mung bean nuclease, or Exonuclease I from *Escherichia coli* may be used to remove the *AHAS1* amplification primers. Preferably, Exonuclease I use used to remove the *AHAS1* amplification primers.

[035] In the third step of the PM1 assay of the invention, the portion of the amplified *AHAS1* gene that contains the site of the PM1 mutation, that is, position 1874 of SEQ ID NOs:1-4, is further amplified in a second amplification step, using a PM1 forward primer and a PM1 reverse primer. The PM1 forward primer and the PM1 reverse primer are complementary to a portion of the *AHAS1* gene within the portion amplified by the *AHAS1* forward primer and the *AHAS1* reverse primer, as depicted in Figure 1, and are thus "nested" within the primers that amplify the *AHAS1* gene. In a preferred embodiment, the PM1 forward primer comprises the sequence 5' CATACCTGTTGGATGTGATAT 3' (SEQ ID NO:11), and the PM1 reverse primer comprises the sequence 5' AAACAACAACAGCGAGTACGT 3' (SEQ ID NO:12). Those of skill in the art will recognize that other primers may be used to selectively amplify the portion of the *B. napus AHAS1* gene which corresponds to the PM1

mutation. In accordance with the invention, the portion of the amplified *AHAS1* gene that contains the site of the PM1 mutation may optionally be labeled using a radioactive tracer, a fluorescent dye, a luminescent label, a paramagnetic label, or any other label suitable for detection of nucleic acids.

[036] In the fourth step of the PM1 assay of the invention, the product of the second amplification step is denatured and placed under conditions that lead to the adoption of a specific single-stranded conformation, dependent on its nucleotide sequence. A variety of methods for denaturing and partial reannealing nucleic acids is known in the art, and any such method may be used in this step of the PM1 assay of the invention. Preferably, the polynucleotides are denatured using heat treatment, for example, exposure to temperatures of 90°C or greater for about ten minutes, and partially renatured by rapid cooling on ice. Alternatively, the polynucleotides containing the site of the PM1 mutation may be denatured using treatment with alkali and partially renatured by addition of acid to reduce the pH.

[037] In the final step of the PM1 assay of the invention, the presence or absence of the PM1 mutation is detected on the basis of the mobility of the polynucleotide conformer in a substrate. Any detection method suitable for separating polynucleotides may be used in this step, for example, gel electrophoresis, high performance liquid chromatography, capillary electrophoresis, and the like. Substrates for such methods are well known, and include, without limitation, polyacrylamide, linear polyacrylamide, poly(N,N-dimethylacrylamide), hydroxyalkyl cellulose, polyoxyethylene, F127 (copolymer of polyoxyethylene and polyoxypropylene, BASF, Ludwigshafen, Germany), agarose, diethylaminoethyl cellulose, sepharose, GENESCAN (Applied Biosystems, Foster City, CA, USA), POP (Amersham Biosciences AB, Uppsala, SE), and the like. When the amplified nucleic acid has been labeled, the detection step may include detection of the radioactive, fluorescent, luminescent, paramagnetic, or other label. When the amplified nucleic acid has not been labeled, detection of the single stranded polynucleotide conformers in the substrate may be performed using known methods, such as silver staining, fluorescent and the like.

[038] In accordance with the invention, the presence of the PM2 mutation may be inferred from resistance to an imidazloinone herbicide applied to the plant or assay of

AHAS activity in the presence of an imidazolinone herbicide. Plants may then be assayed using the PM1 assay set forth above to determine whether the plant exhibits commercially relevant imidazolinone tolerance.

[039] Alternatively, the plant may be assayed for the presence of the PM2 mutation using the PM2 assay method of the invention, in which the *AHAS3* gene is selectively amplified from isolated genomic DNA in a first amplification step. For this step, an *AHAS3* forward primer and an *AHAS3* reverse primer is combined with the genomic DNA and subjected to PCR amplification as described above. A preferred *AHAS3* forward primer for use in this method of the invention comprises the sequence 5' CACAAGCCTCGTGTTATAAAAA 3' (SEQ ID NO:13), and a preferred *AHAS3* reverse primer comprises the sequence 5' CATTGAGTGCCAAACATTATGTA 3' (SEQ ID NO:14). Those of skill in the art will recognize that other primers may be used to selectively amplify the *B. napus AHAS3* gene.

[040] After the first amplification step, the *AHAS3* amplification reaction product or mixture is purified to remove the *AHAS3*-specific amplification primers. Any or the purification methods described above may be used for this step. Preferably, Exonuclease I is used to remove the *AHAS3* amplification primers.

[041] After the purification step, the amplified *AHAS3*-containing DNA is divided into at least two aliquots, each of which is separately amplified in the region of the *AHAS3* gene surrounding position 1712 of SEQ ID NOs:5-8, hereinafter referred to as the "PM2 region". A first aliquot of the amplified *AHAS3* DNA is further amplified in a second amplification step, using at least one primer which is selective for a portion of the *AHAS3* gene that is wild type at position 1712 of said gene, that is, which comprises a "G" residue at said position 1712, as depicted in SEQ ID NOs:5 and 8. In a preferred embodiment, the second amplification step employs a wild type-selective forward primer in combination with a forward and reverse primers that selectively amplify the PM2 region. All three of these primers are nested within the primers employed to amplify the *AHAS3* gene in the first amplification step. In a preferred embodiment, the forward primer for amplification of the PM2 region comprises the sequence 5' ACTCGGAGCTATGGGTTTC 3' (SEQ ID NO:15), and the reverse primer for amplification of the PM2 region comprises the sequence 5' ATCCAACAGGTACGGTCCA 3' (SEQ ID NO:16), the wild type selective primer

comprises the sequence 5' TGGGATGGTCATGCAATG 3' (SEQ ID NO:17). Those of skill will recognize that other primers may be used to amplify the PM2 region.

[042] In accordance with the invention, a second aliquot of the amplified and purified *AHAS3*-containing DNA is further amplified in a third amplification step, using at least one primer which is selective for the PM2 mutation, that is, which comprises a "T" residue at said position 1712 of the *AHAS3* gene, as depicted in SEQ ID NOs:6 and 7. In a preferred embodiment, the second amplification step employs a PM2-selective forward primer in combination with a forward and reverse primers that selectively amplify the PM2 region. All three of these primers are nested within the primers employed to amplify the *AHAS3* gene in the first amplification step. In a preferred embodiment, the PM2-selective primer comprises the sequence 5' CTTGGGATGGTCATGCAATT 3' (SEQ ID NO:18), the forward primer for amplification of the PM2 region comprises the sequence 5' ACTCGGAGCTATGGGTTTC 3' (SEQ ID NO:16), and the reverse primer for amplification of the PM2 region comprises the sequence 5' ATCCAACAGGTACGGTCCA 3' (SEQ ID NO:17). Those of skill will recognize that other primers may be used to amplify the PM2 region.

[043] The second and third amplification steps may be performed iteratively or simultaneously.

[044] In accordance with the invention, in the second and third amplification steps, the portion of the amplified *AHAS3* gene that contains the site of the PM2 mutation may optionally be labeled using a radioactive tracer, a fluorescent dye, a luminescent label, a paramagnetic label, or any other label suitable for detection of nucleic acids.

[045] In the final step of the PM2 assay of the invention, the products of the second and third amplification steps are analyzed for the presence or absence of the PM2 mutation using known methods, such as gel electrophoresis, high performance liquid chromatography, capillary electrophoresis, and the like. When the amplified nucleic acids have been labeled, the analysis step may include detection of the radioactive, fluorescent, luminescent, paramagnetic, or other label. When the amplified nucleic acids have not been labeled, the analysis step may be performed using known methods, such as ethidium bromide staining, and the like.

[046] The invention is also embodied in isolated nucleic acids which are formed as reaction products of the amplifications described herein. In one embodiment, the nucleic acid reaction product corresponds to the region of the *AHAS1* gene between the *AHAS1* forward amplification primer and the *AHAS1* reverse amplification primer, and has a sequence as set forth from nucleotide 96 to nucleotide 2330 of SEQ ID NO:19. In another embodiment, the nucleic acid reaction product corresponds to the region of the *AHAS1* gene between the PM1 forward primer and the PM1 reverse primer and is exemplified by a sequence as set forth from nucleotide 1817 to nucleotide 2063 of SEQ ID NO:1; a sequence as set forth from nucleotide 1735 to nucleotide 1980 of SEQ ID NO:2; a sequence as set forth from nucleotide 1809 to nucleotide 2054 of SEQ ID NO:3; and a sequence as set forth from nucleotide 1720 to nucleotide 1966 of SEQ ID NO:4.

[047] In another embodiment, the nucleic acid reaction product corresponds to the region of the *AHAS3* gene between the *AHAS3* forward amplification primer and the *AHAS3* reverse amplification primer, and has a sequence as set forth from nucleotide 64 to nucleotide 2310 of SEQ ID NO:20. The invention is further embodied by a nucleic acid corresponding to the PM2 region of the *AHAS3* gene, between the PM2 forward primer and the PM2 reverse primer. Examples of these reaction products include nucleic acids having a sequence as set forth from nucleotide 1383 to nucleotide 1770 of SEQ ID NO:5; nucleic acids having a sequence as set forth from nucleotide 1518 to nucleotide 1905 of SEQ ID NO:6; nucleic acids having a sequence as set forth from nucleotide 1352 to nucleotide 1739 of SEQ ID NO:7; and nucleic acids having a sequence as set forth from nucleotide 1308 to nucleotide 1695 of SEQ ID NO:8. Additional nucleic acids are encompassed in this embodiment as the reaction products of the third amplification reaction of the PM2 assay, that is, nucleic acids having a sequence as set forth from nucleotide 1560 to nucleotide 1770 of SEQ ID NO:5; nucleic acids having a sequence as set forth from nucleotide 1695 to nucleotide 1905 of SEQ ID NO:6; nucleic acids having a sequence as set forth from nucleotide 1529 to nucleotide 1739 of SEQ ID NO:7; and nucleic acids having a sequence as set forth from nucleotide 1485 to nucleotide 1695 of SEQ ID NO:8.

[048] The PM1 and PM2 assays, oligonucleotides, and nucleic acid reaction products may also be used in a marker assisted breeding program to make progeny

canola plants by selective breeding. In such a program, other markers in addition to the PM1 and PM2 polymorphisms would be required, as is known in the art. Methods of marker assisted selection are described, for example, in U.S.Pat.No. 6,100,030.

[049] The invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof.

EXAMPLE 1

Materials and Genomic DNA Isolation

[050] The canola lines used for these experiments are listed in Table 1 below. The nucleic acid sequences of the *AHAS1* genes from each of these lines are shown in Figure 1, and the nucleic acid sequences of the *AHAS3* genes for each of these lines are shown in Figure 2.

Table 1

Lines	Mutations	Code	Herbicide resistance
T9107	Point mutation 1 on AHAS1	PM1	Partial resistant
T9108	Point mutation 2 on AHAS3	PM2	Partial resistant
TR101	Point mutation 1 + 2	R	Resistant
OPTION 501	wild type	S	Susceptible

[051] Plants were grown from seeds of each canola line. Three to five leaf punches from each plant were combined in each sample, and samples were freeze dried. The freeze dried samples were ground by adding cleaned BB's (BB's were washed with soap and water and then dried with organic solvent prior to use) to each sample and shaking the samples until a fine powder was obtained (approximately one minute). Five hundred μl of Extraction Buffer (1300 μl 1M Tris; 4.15 ml dd H_2O; 325 μl 0.5M EDTA; 650 μl 10% SDS) was added to each sample, and the samples were inverted several times. The samples were then placed into a 65°C water bath for 60 minutes, with inversions every 20 minutes. During the sample incubation, a second set of test tubes was filled with 400 μl isopropanol.

[052] After the incubation period, the samples were allowed to cool for 5 minutes and centrifuged briefly in a microfuge. Five μl RNAase A (10 mg/ml) was added to each sample tube, and the tubes were inverted about 20 times. The samples were again centrifuged briefly in a microfuge and allowed to sit at room temperature for 30 minutes. To each sample was added 170 μl 7.5M ammonium acetate, and samples were shaken for approximately 2 minutes to precipitate protein. The samples were then centrifuged briefly in a microfuge, placed on ice for 15 minutes, and then re-centrifuged for 15 minutes. The supernatants were retained and placed into the previously prepared isopropanol-containing test tubes, which were then gently

inverted approximately 50 times to precipitate DNA. The sample tubes were then centrifuged at maximum rpm for 15 minutes. The supernatants from this centrifugation were discarded, and the DNA pellets were washed once with 300 μl 95% ethanol and twice with 300 μl 70% ethanol. After being allowed to dry overnight, the washed DNA pellets were resuspended in 50 μl ddH_2O for further analysis.

EXAMPLE 2

PM1 Assay

[053] A single strand conformational polymorphism (SSCP) analysis was carried out by denaturing products of two rounds of PCR which selectively amplified the region of the *Brassica AHAS1* gene that corresponds to the PM1 mutation, that is, the region surrounding position 1874 of SEQ ID NOs:1-4, and allowing each of the single strands to reanneal partially with itself. The conformation of each of the single strands, along with its nucleotide sequence, determines its mobility in a non-denaturing gel.

A. *AHAS1*-Specific Amplification Step

[054] The conditions used for the first round of PCR amplification are listed in Table 2. The *AHAS1*-specific forward primer used for the first amplification step had the sequence
5' CACAAGTCTCGTGTTATAAAAC 3' (SEQ ID NO:9) and the *AHAS1*-specific reverse primer used had the sequence 5' CATTGAGTGCCAAACATATGAA 3' (SEQ ID NO:10). A Tetrad thermocycler (MJ Research) was used for PCR amplification. The first round PCR reactions consisted of an initial denature of 5 minutes at 94°C followed by 25 cycles (30 seconds at 94°C, 1 minute at 60°C, 1 minute at 72°C), with a final extension of 10 minutes at 72°C. To 2 μl of the product of the first round of amplification, 0.5 unit ExoI (Exonuclease I) was added and incubated at 37°C for 1 hour before the enzyme was inactivated at 72°C for 15 minutes. 100 μL of deionized distilled water (ddH_2O) was added following the ExoI reaction.

Table 2

	Final concentration
Genomic DNA	± 10 ng
Buffer (BRL - 10 x)	1 x
$MgCl_2$ (BRL - 50 mM)	2.5 mM
dNTPs	0.2 mM
Primer forward	0.5 μM
Primer reverse	0.5 μM
Taq (BRL - 5 U/μl)	0.4 U
H_2O	→ 15 μl

B. PM1-Specific Amplification Step

[055] The conditions used for the second round of the PCR amplification are shown in Table 3. The reaction consisted of an initial denature of 5 min at 94°C followed by 35 cycles (30 seconds at 94°C, 1 minute at 65°C, 1 minute at 72°C), with a final extension of 10 minutes at 72°C. The PM1-specific primers are nested within the primers used to amplify the *AHAS1* gene and thus specifically amplify the region surrounding position 1874 of SEQ ID NOs:1-4. The PM1-specific forward primer used in the second amplification step had the sequence 5' CATACCTGTTGGATGTGATAT 3' (SEQ ID NO:11), and the PM1-specific reverse primer had the sequence 5' AAACAACAACAGCGAGTACGT 3' (SEQ ID NO:12).

Table 3

	Final concentration
Diluted *AHAS1* PCR product	1 μl
Buffer (BRL - 10 x)	1 x
$MgCl_2$ (BRL - 50 mM)	2 mM
dNTPs	0.2 mM
Primer forward	0.5 μM
Primer reverse	0.5 μM
Taq (BRL - 5 U/μl)	0.4 U
H_2O	→ 15 μl

C. SSCP Analysis of PM1 Amplification Products

[056] An 8 M urea stop solution containing bromo phenol blue, xylene cyanol and Orange G tracking dyes was added to a final concentration of 5M. The mixtures were

denatured for 10 minutes at 90°C and quickly cooled on ice. The SSCPs were electrophoresed on a 12 % non-denaturing acrylamide/bisacrylamide (49:1) in 0.5 x Tris borate EDTA (TBE) buffer. The gels were run at constant amperage of 17 mA for 20-24 hours at 4°C. The DNA was visualized by silver staining. The resulting gel clearly and accurately identified the presence or absence of imidazolinone resistant (PM1) and susceptible (wild type) alleles for all tested strains.

EXAMPLE 3

PM2 Assay

[057] This assay employed a first round of PCR which selectively amplifies the *AHAS3* gene, after which the amplification product was divided into two aliquots. Each aliquot was then amplified separately, using sets of three primers nested within those used for amplifying the *AHAS3* gene. The three primers selectively amplify the region of the *AHAS3* gene corresponding to the PM2 mutation, that is, the region surrounding position 1712 of SEQ ID NOs; 5-8. Separate PCR steps were performed on each aliquot, one which selectively amplifies nucleic acids containing the PM2 mutation and one which selectively amplifies wild type nucleic acids. The presence of wild type or PM2 was detected by gel electrophoresis.

A. *AHAS3*-Specific Amplification Step

[058] The conditions used for the first round of amplification are shown in Table 4. The *AHAS3*-specific forward primer used for the first amplification step had the sequence 5' CACAAGCCTCGTGTTATAAAAA 3' (SEQ ID NO:13), and the *AHAS3*-specific reverse primer had the sequence 5' CATTGAGTGCCAAACATTATGTA 3' (SEQ ID NO:14). The PCR reactions consisted of an initial denature of 5 minutes at 94°C followed by 25 cycles (30 seconds at 94°C, 1 minute at 60°C, 1 second at 72°C), with a final extension of 10 minutes at 72°C. To 2 µl of this PCR product, 0.5 unit ExoI was added and incubated at 37°C for 1 hour before the enzyme was inactivated at 72°C for 15 minutes. 100 µL of ddH20 was added following the ExoI reaction.

Table 4

	Final concentration
Genomic DNA	± 10 ng
Buffer (BRL - 10 x)	1 x
$MgCl_2$ (BRL - 50 mM)	2.5 mM
dNTPs	0.2 mM
Primer forward	0.5 μM
Primer reverse	0.5 μM
Taq (BRL - 5 U/μl)	0.4 U
H_2O	→ 15 μl

B. PM2 Region-Specific Amplification Steps

[059] The conditions used for the second round of the nested PCR with the different primer sets are described in Table 5. The PM2 region-specific primers are nested within the primers used to amplify the *AHAS3* gene and thus specifically amplify the region surrounding position 1712 of SEQ ID NOs:5-8. The PM2 region-specific forward primer (PM2 F in Table 5) had the sequence 5' ACTCGGAGCTATGGGTTTC 3' (SEQ ID NO:15), and the PM2 region-specific reverse primer (PM2 R in Table 5) had the sequence 5' ATCCAACAGGTACGGTCCA 3' (SEQ ID NO:16). The amplification primer specific for the wild type allele at position 1712 (PM2 sus in Table 5) had the sequence 5' TGGGATGGTCATGCAATG 3' (SEQ ID NO:17), and the primer specific for the PM2 mutation (PM2 res in Table 5) had the sequence 5' CTTGGGATGGTCATGCAATT 3' (SEQ ID NO:18). The cycling conditions for the second and third amplification steps were as follows: an initial denature of 5 min at 94°C followed by 38 cycles (30 at seconds 94°C, 45 seconds at 65°C, 60 seconds at 72°C), with a final extension of 10 minutes at 72°C.

Table 5

	Wild type	PM2
Diluted *AHAS3* PCR product	1 μl	1 μl
Buffer (BRL - 10 x)	1 x	1 x
$MgCl_2$ (BRL - 50 mM)	2 mM	2 mM
dNTPs	0.2 mM	0.2 mM
PM2 F	0.5 μM	0.5 μM
PM2 res		0.5 μM
PM2 sus	0.5 μM	
PM2 R	0.5 μM	0.5 μM
Taq (BRL - 5 U/μl)	0.4 U	0.4 U
H_2O	→ 15 μl	→ 15 μl

[060] After amplification was complete, 6x loading buffer was added to all reactions, (4g sucrose, 2.4 mL 0.5M EDTA, bromophenol blue, xylene cyanol and Orange G to final 10 mL volume). The products of the second and third amplification steps were run on a 3.5 % metaphor gel for 4 hours at 92 V. Each amplification reaction yielded two PCR fragments: a larger PCR fragment resulting from the PM2 region specific primers and a smaller PCR fragment created by amplification of the wild type-specific primer or PM2-specific primer in combination with the reverse PM2 region specific primer. The larger fragment was used as a positive control for the PCR reaction. The smaller PCR fragment was an allele-specific PCR product. The gel clearly and accurately identified the presence or absence of imidazolinone resistant (PM2) and susceptible (wild type) alleles for all tested strains.